

DESCRIPTION

MEMBRANE PREPARATION FROM *PICHLA PASTORIS* TO ASSAY γ -SECRETASE ACTIVITY

BACKGROUND OF THE INVENTION

This application claims the priority of U.S. Provisional Patent Application Serial No. 60/505,601, filed September 24, 2003, the entire disclosure of which is specifically incorporated herein by reference.

1. Field of the Invention

The present invention relates generally to screening methods for identifying molecules that interact with and regulate proteins of interest. More particularly, it concerns the development of screening methods to identify molecules that inhibit γ -secretase activity.

2. Description of Related Art

Alzheimer's disease (AD) is the most common type of progressive dementia in the elderly. AD is characterized by initial memory loss, followed by progressive loss of neurons leading to dementia and loss of all nervous functions, and eventually death. AD is now the fourth-largest killer of adults 65 and older, and this disease impacts about one of every three families in the United States, and affects over 13 million people world-wide. As the population trends lead to an increase in the number of older people, this figure will only increase.

A subset of AD is classified as familial early-onset AD (FAD) where onset of the disease begins as early as the fourth to sixth decade of life. FAD is an inherited autosomal dominant disorder. Mutations in genes encoding polytopic membrane proteins called presenilins (PS), exemplified by PS1 and PS2, co-segregate with the majority of pedigrees with early-onset AD. PS1 mutations known to associate with FAD include M146L, E280A, and G384A.

AD is pathologically characterized by the presence of neurofibrillary tangles and the cerebral deposition of β -amyloid ($A\beta$) peptides that are 40-42 amino acids in length and are derived from the amyloid precursor protein (APP). Two lines of evidence demonstrate the involvement of PS (PS1 and PS2) in $A\beta$ production. First, $A\beta$ production is abrogated in PS1 deficient (PS1^{-/-}) cells. Second, FAD-linked mutant PS1 increases the production of highly fibrillogenic $A\beta$ 42 peptides. The PS proteins mediate the cleavage of APP and also facilitate

proteolytic processing of other transmembrane (TM) proteins such as APP homologues, APLP1, Notch 1, Irel, and likely other yet unidentified proteins. However, the precise role of PS1 in A β production, and the molecular mechanisms by which FAD-linked PS1 mutations lead to elevations in A β 42 production are not yet defined. In addition, very little information is available regarding the molecular and structural domains of PS1 critical for these biological functions.

PS1 (SEQ ID NO:1, nucleotide; SEQ ID NO:2, amino acid), a 467 amino acid polytopic membrane protein, accumulates as ~30 kDa N-terminal (NTF) and ~20 kDa C-terminal (CTF) derivatives in mammalian cells and tissues. The conversion of PS1 holoprotein to the activated NTF/CTF form is necessary for γ -secretase activity. The NTF and CTF are the "functional" units, and are known to associate with additional membrane proteins, termed nicastrin, APh-1 and PEN-2 in high molecular weight complexes. The accumulation of each of these constituents is dependant on the expression of other components of the complex.

A series of biochemical and genetic studies have confirmed that presenilin 1 (PS1) plays a critical role in facilitating intramembranous γ -secretase processing of β -amyloid precursor protein (APP) and several additional transmembrane proteins. γ -secretase-mediated processing of APP liberates small ~4 kDa peptides, termed A β , that are fibrillogenic and neurotoxic and the principal components of amyloid plaques in Alzheimer's disease.

It has been recently demonstrated by Edbauer *et al.* that γ -secretase activity can be reconstituted in *Saccharomyces cerevisiae*, an organism lacking endogenous γ -secretase activity, by co-expressing PS1, nicastrin, APh-1, and PEN-2. Nucleotide and amino acid sequences for any of these genes can readily be obtained by methods known to those of skill in the art from publications and computer databases such as Genbank. While Edbauer *et al.* provides proof of principle that γ -secretase activity can be reconstituted in an amenable model system, it fails to suggest that such a system could be used to identify compounds that modulate γ -secretase activity nor does it suggest any parameters that could be changed to enhance the system's ability to identify γ -secretase modulators. The identification of compounds that modulate γ -secretase activity will be of great benefit in understanding the molecular basis for AD, and will lead to better and more effective treatments for AD.

A more robust and reproducible system is needed to more effectively identify compounds that affect γ -secretase activity.

SUMMARY OF THE INVENTION

The present invention overcomes the above-described deficiencies in the art and provides a transgenic *Pichia pastoris* and screening methods for the identification of compounds that modulate γ -secretase activity. The inventors have generated *P. pastoris* strains that express PS1, APH-1, nicastrin, and PEN-2 individually, and in all possible combinations. The inventors have generated similar *P. pastoris* strains in which PS1 is epitope tagged with a Tandem Affinity Purification tag (TAP) that allows the purification of the complex away from other proteins present in the solubilized membrane preparation. The inventors have also generated a *P. pastoris* strain that expresses PS1, APH-1, nicastrin, PEN-2, and APP C99.

The invention provides a transgenic *Pichia pastoris* comprising at least one of a presenilin 1, APH-1, nicastrin, or PEN-2 encoding transgene. Preferably the transgenic *P. pastoris* comprises presenilin 1, APH-1, nicastrin, and PEN-2 encoding transgenes. More preferably, the transgenic *P. pastoris* comprising presenilin 1, APH-1, nicastrin, and PEN-2 encoding transgenes exhibits γ -secretase activity. In some embodiments the transgenic *P. pastoris* further comprises a transgene encoding amyloid precursor protein (APP) or a derivative of APP. In a preferred embodiment, the APP derivative is a carboxyl-terminal derivative termed C99. One or more of presenilin 1, APH-1, nicastrin, PEN-2, and APP may be epitope tagged. In a preferred embodiment, presenilin 1 is epitope tagged. More, particularly, the epitope tag is a Tandem Affinity Purification (TAP) tag.

In some embodiments, presenilin 1 is a mutant presenilin 1. Preferably, the mutant presenilin 1 contains a mutation associated with FAD. In some embodiments of the invention, the presenilin 1 mutation is M146L, E280A, or G384A. In some aspects of the invention the mutant presenilin 1 is epitope tagged.

In other embodiments, the invention provides a transgenic *P. pastoris* comprising APH-1, nicastrin, PEN-2, and mutant presenilin 1 encoding transgenes, wherein the *P. pastoris* exhibits no γ -secretase activity. In particular embodiments, the mutant presenilin 1 is D385A. In some embodiments, the mutant presenilin 1 is epitope tagged.

Of course, those of skill in the art would understand that mutations in APP, APH-1, nicastrin, or PEN-2 could be studied using the methods and compositions of the present invention. Those of skill in the art would also understand that PS2 could be studied using the methods and compositions of the present invention.

In other aspects, the invention provides methods of producing a transgenic *P. pastoris* with γ -secretase activity comprising providing the *P. pastoris* with a transgene encoding presenilin 1, APH-1, nicastrin, and PEN-2.

In some embodiments, the invention provides methods of identifying compounds that
5 inhibit γ -secretase activity. In one embodiment, the invention provides a method of identifying a compound that inhibits γ -secretase comprising: a) preparing a solubilized membrane preparation from a transgenic *P. pastoris* comprising presenilin 1, APH-1, nicastrin, and PEN-2 encoding transgenes, wherein the *P. pastoris* exhibits γ -secretase activity; b) contacting the solubilized membrane preparation with a substrate for γ -secretase and a test compound; and c) determining
10 whether γ -secretase activity is decreased in the presence of the test compound, the decrease in γ -secretase activity being an indication that the test compound inhibits γ -secretase activity. In a preferred embodiment, the substrate for γ -secretase is β -amyloid precursor protein or a derivative of β -amyloid precursor protein.

In other embodiments, the invention provides methods of identifying a compound that
15 inhibits γ -secretase activity comprising: a) preparing a solubilized membrane preparation from a transgenic *P. pastoris* comprising presenilin 1, APH-1, nicastrin, and PEN-2 encoding transgenes, wherein the *P. pastoris* exhibits γ -secretase activity; b) isolating γ -secretase away from the solubilized membrane preparation; c) contacting the isolated γ -secretase with a substrate for γ -secretase and a test compound; and d) determining whether γ -secretase activity is
20 decreased in the presence of the test compound, the decrease in γ -secretase activity being an indication that the test compound inhibits γ -secretase activity. In a preferred embodiment, the substrate for γ -secretase is β -amyloid precursor protein or a derivative of β -amyloid precursor protein.

In yet other embodiments, the invention provides methods of identifying a compound that
25 inhibits γ -secretase activity comprising: a) contacting a transgenic *P. pastoris* comprising presenilin 1, APH-1, nicastrin, PEN-2, and APP C99 encoding transgenes, wherein the *P. pastoris* exhibits γ -secretase activity, with a test compound; and b) determining whether γ -secretase activity is decreased in the presence of the test compound, the decrease in γ -secretase activity being an indication that the test compound inhibits γ -secretase activity.

30 The invention also provides methods of treating Alzheimer's disease. In one embodiment, the invention provides methods of treating Alzheimer's disease comprising: a) identifying a patient suffering from or at risk for Alzheimer's disease; and b) administering to the patient a compound that inhibits γ -secretase activity, wherein the compound is identified by a

method comprising: preparing a solubilized membrane preparation from a transgenic *P. pastoris* comprising presenilin 1, APH-1, nicastrin, and PEN-2 encoding transgenes, wherein the *P. pastoris* exhibits γ -secretase activity; contacting the solubilized membrane preparation with a substrate for γ -secretase and a test compound; and determining whether γ -secretase activity is decreased in the presence of the test compound, the decrease in γ -secretase activity being an indication that the test compound inhibits γ -secretase activity.

In other embodiments, the invention provides methods of treating Alzheimer's disease comprising: a) identifying a patient suffering from or at risk for Alzheimer's disease; and b) administering to the patient a compound that inhibits γ -secretase activity, wherein the compound is identified using a method comprising: preparing a solubilized membrane preparation from a transgenic *Pichia pastoris* comprising presenilin 1, APH-1, nicastrin, and PEN-2 encoding transgenes, wherein the *Pichia pastoris* exhibits γ -secretase activity; isolating γ -secretase away from the solubilized membrane preparation; contacting the isolated γ -secretase with a substrate for γ -secretase and a test compound; and determining whether γ -secretase activity is decreased in the presence of the test compound, the decrease in γ -secretase activity being an indication that the test compound inhibits γ -secretase activity.

In other embodiments, the invention provides methods of treating Alzheimer's disease comprising: a) identifying a patient suffering from or at risk for Alzheimer's disease; and b) administering to the patient a compound that inhibits γ -secretase activity, wherein the compound is identified using a method comprising: contacting a transgenic *P. pastoris* comprising presenilin 1, APH-1, nicastrin, PEN-2, and APP C99 encoding transgenes, wherein the *P. pastoris* exhibits γ -secretase activity, with a test compound; and determining whether γ -secretase activity is decreased in the presence of the test compound, the decrease in γ -secretase activity being an indication that the test compound inhibits γ -secretase activity.

In some embodiments, the compound identified according to a method of the present invention is an aspartyl protease inhibitor. In certain aspects of the invention, the compound identified according to a method of the present invention is L685,458, pepstatin, pepstatin methylester, pepstatin A, JKL2, aryksykfibanude 1, benzodiazepine 2 (compound E), compound WPE-III-31C, DAPT {N-[N-(3,5-di-uorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester}, L852,646, NVP-AHW700-NX, or an analog of one of the above-mentioned compounds. An "analog" refers to a structural derivative of a parent chemical compound that often differs from the parent compound by a single element. The difference between the structural derivative and

the parent compound may be, for example, the replacement of one atom by an atom of a different element or the presence or absence of a particular functional group.

As used herein the specification, "a" or "an" may mean one or more. As used herein in the claim(s), when used in conjunction with the word "comprising", the words "a" or "an" means
5 one or more than one. As used herein "another" may mean at least a second or more.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit
10 and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

15 The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

20 **FIG. 1:** The expression of PS1, APH-1, PEN-2, and nicastrin in *P. pastoris*.

FIG. 2: The conversion of PS1 holoprotein to PS1 NTF and PS1 CTF in the presence of APH-1, PEN-2, and nicastrin.

FIG. 3 Reconstitution of γ -secretase activity in soluble membrane preparations from *P. pastoris*. Inhibition of γ -secretase activity in soluble membrane preparations from *P. pastoris*
25 with 1 μ M L685,458.

FIG. 4A and FIG. 4B: *In vivo* γ -secretase activity in *P. pastoris*.

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

I. The Present Invention

A. Presenilins and Presenilin Metabolism

5 PS1 is a 467 amino acid polypeptide predicted to contain 8 transmembrane (TM) spanning domains with its N-terminus, C-terminus and a large hydrophilic "loop" region located between TMs 6 and 7 all oriented towards the cytosol. Although PS1 is synthesized as a 42- to 43-kD polypeptide, the preponderant PS1-related species that accumulate *in vivo* are the N-terminal (NTF) and the C-terminal (CTF) proteolytic derivatives. These PS1 derivatives are
10 generated by endoproteolysis at Met 292 (and to a lesser extent, Met 298) within the cytoplasmic "loop" domain between TMs 6 and 7. In addition to the endoproteolytic processing described above, PS1 and PS2 also undergo additional cleavage within the hydrophilic loop domain, dubbed "alternative cleavage," that is mediated by caspases.

Presenilins (PS), including PS1 and PS2, are involved in the pathogenesis of Alzheimer's
15 Disease (AD) and mutations in presenilins have been associated in ~50% of pedigrees with familial early-onset AD (FAD). For example, PS1 is necessary for γ -secretase cleavage of the Alzheimer's precursor protein (APP) and during APP cleavage FAD-linked PS1 and PS2 mutants have been shown to selectively enhance the production of the A β 42 peptides in transfected mammalian cells, in the brains of transgenic mice, as well as in patients with AD
20 (Borchelt *et al.*, 1996; Duff *et al.*, 1996; Scheuner *et al.*, 1996; Tomita *et al.*, 1997; Borchelt *et al.*, 1997; Holcomb *et al.*, 1998). The A β 42 peptides are more fibrillogenic than the shorter A β 40 peptides and thus, FAD-linked mutant PS proteins lead to the production of highly amyloidogenic A β species which leads to A β deposition in brain.

B. FAD-linked PS1 mutations influence APP metabolism

25 The mechanisms by which FAD-linked PS variants cause AD are not fully understood, but several important insights have emerged. The most provocative insight came from studies that demonstrated that FAD-linked PS1 and PS2 variants selectively enhance the production of A β 42 peptides in transfected mammalian cells, the brains of transgenic mice, and patients with
30 AD (Borchelt *et al.*, 1996; Duff *et al.*, 1996; Scheuner *et al.*, 1996; Tomita *et al.*, 1997; Borchelt *et al.*, 1997; Holcomb *et al.*, 1998). A β 42 peptides are more fibrillogenic than the shorter A β 40 peptides, and are more prominent in the amyloid lesions of patients with AD (Jarrett *et al.*, 1993; Iwatsubo *et al.*, 1994; Gravina *et al.*, 1995). Thus, while PS 1 is necessary for the γ -secretase

cleavage of APP, FAD-linked mutant PS bias this cleavage toward the production of highly amyloidogenic A β 42 species that foster A β deposition in brain. The mechanism(s) by which mutant PS influences the production of A β 42 peptides are uncertain, but FAD-linked mutant PS proteins appear to cause aberrant gain, rather than loss, of function (Sisodia *et al.*, 1999).

5 Understanding how PS proteins influence the production of A β 42 peptides is of central importance to AD research.

C. Presenilins Facilitate Intramembranous Processing of APP

Analysis of APP processing in neurons from mice containing a targeted deletion of PS1 revealed that PS1 is required for γ -secretase cleavage of APP; PS1 deficiency is associated with defects in the secretion of A β peptides and intracellular accumulation of APP C-terminal fragments (CTFs) bearing varying extents of the A β region (De Strooper *et al.*, 1998; Naruse *et al.*, 1998). Wolfe and colleagues presented evidence for two critical aspartate residues within TMs 6 and 7 of PS1 that play an important role in γ -secretase processing of APP (Wolfe *et al.*, 1999); mutation at either of the aspartate residues leads to substantial reductions in A β secretion and accumulation of APP CTFs. Regardless of whether PS is a critical co-factor for γ -secretase or itself is the γ -secretase, it is remarkable that FAD-linked PS that harbors independent mutations at multiple TMs and linker domains can specifically influence the generation of A β 42 peptides, despite the fact that PS1 is required for the production of both A β 40 and A β 42. Thus, the connection between FAD-linked PS mutations, its influence on the γ -secretase and enhanced production of A β 42 is far from clear.

D. Screening for Inhibitors of γ -Secretase Activity

The present invention provides methods for identifying inhibitors of γ -secretase activity. The screening assays may comprise random screening of large libraries of test compounds. Alternatively, the assays may be used to focus on particular classes of compounds selected with an eye towards structural attributes that are believed to make them more likely to modulate γ -secretase activity. Thus, the screening assays will assay for increases or decreases in levels of γ -secretase activity in response to a test compound.

Alternatively, one may assay for a change in function or activity of PS1, APH-1, nicastrin, and PEN-2. The change may be an increase or a decrease in activity or function of the protein. By function, it is meant that one may assay for any protein related biological activity, such as an increased/decreased enzyme activity, an increased/decreased electrical activity

corresponding to increased/decreased levels of ion channels, transcriptional activity measured directly or via promoter assays (CAT assays or luciferase assays), Ca^{++} imaging, cell surface expression of marker proteins, cell survival or cell death, *etc.* For example, one may assay the conversion of PS1 holoprotein to NTF/CTF.

5 To identify a compound that inhibits γ -secretase, one generally will determine the level of γ -secretase activity in the presence and absence of a test compound. For example, in some embodiments the methods of the invention comprise:

a) preparing a solubilized membrane preparation from *Pichia pastoris* with γ -secretase activity;

10 b) contacting the solubilized membrane preparation with a substrate for γ -secretase and a test compound; and

c) determining whether γ -secretase activity is decreased in the presence of the test compound, the decrease in γ -secretase activity being an indication that the test compound inhibits γ -secretase activity.

15 In other embodiments, the methods of the invention comprise:

a) contacting a transgenic *P. pastoris* having γ -secretase activity and expressing APP C99 with a test compound; and

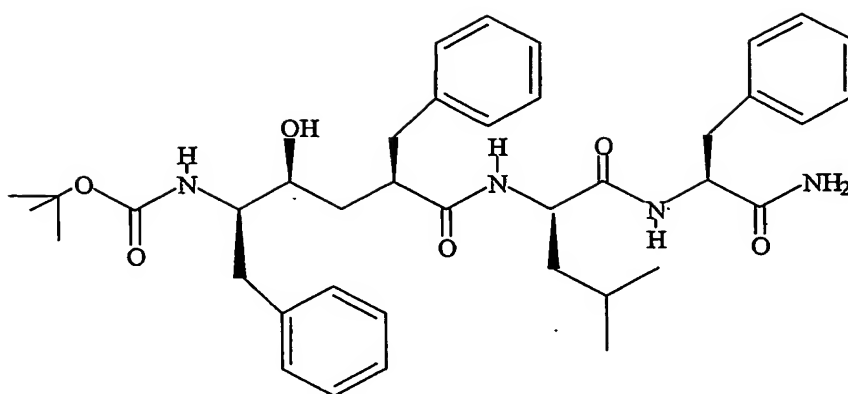
b) determining whether γ -secretase activity is decreased in the presence of the test compound, the decrease in γ -secretase activity being an indication that the test compound
20 inhibits γ -secretase activity.

a. Inhibitors

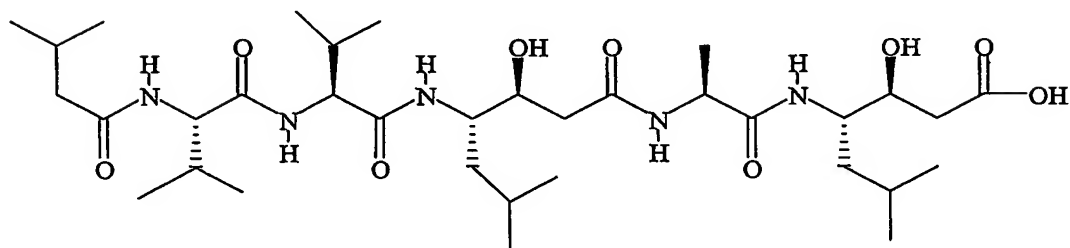
As used herein the term "test compound" refers to any molecule that may potentially inhibit γ -secretase activity. The test compound may be a protein or fragment thereof, a small
25 molecule, or even a nucleic acid molecule. It may prove to be the case that the most useful pharmacological compounds will be compounds that are structurally related to PS1, A β -1, nicastrin, PEN-2, or APP. It may also prove to be the case that the most useful pharmacological compounds will be compounds that are structurally related to a substrate of γ -secretase.

There are several known γ -secretase inhibitors. L685,458, an aspartyl protease inhibitor,
30 is a specific γ -secretase inhibitor. Other γ -secretase inhibitors include: pepstatin, pepstatin methylester, pepstatin A, JKL2, aryksykfibanude 1, benzodiazepine 2 (compound E), compound WPE-III-31C, DAPT {N-[N-(3,5-di-uorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester}, L852,646, and NVP-AHW700-NX. Useful pharmacological compounds will include

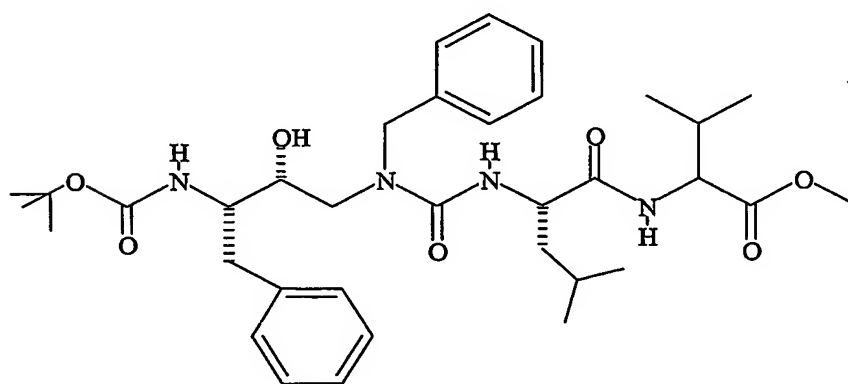
compounds that are, or are structurally or functionally related to, known γ -secretase inhibitors, which have the structures set forth below. Of course, those of skill in the art will be able to take these known structures and modify them to obtain derivatives or design mimetics thereof and test such derivatives and mimetics to prove their activity in the context of the invention by following the teachings of this specification. These listed inhibitors, and analogs, derivatives, and mimetics thereof are exemplary of the inhibitors of the invention.



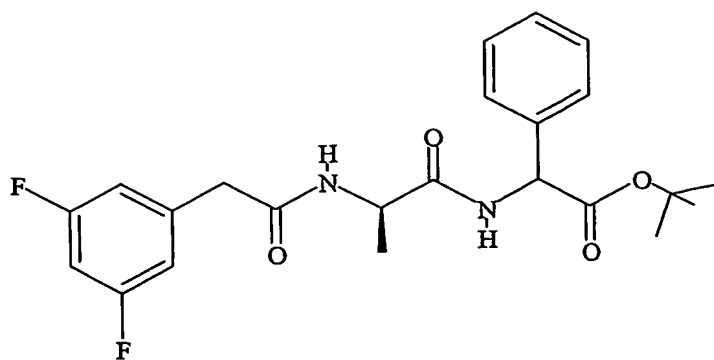
L685,458



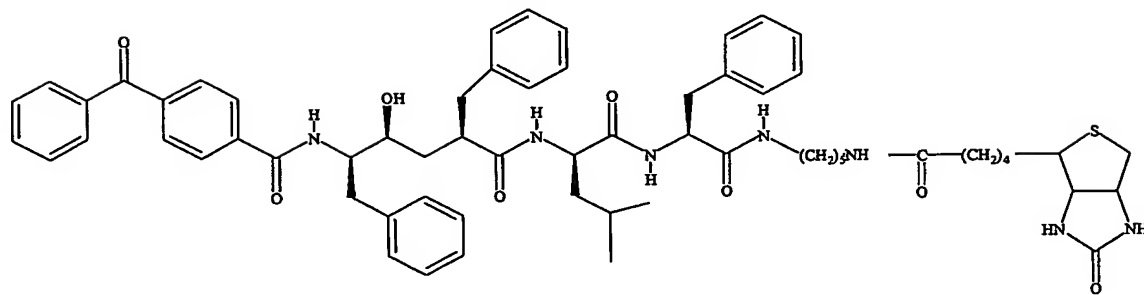
Pepstatin A



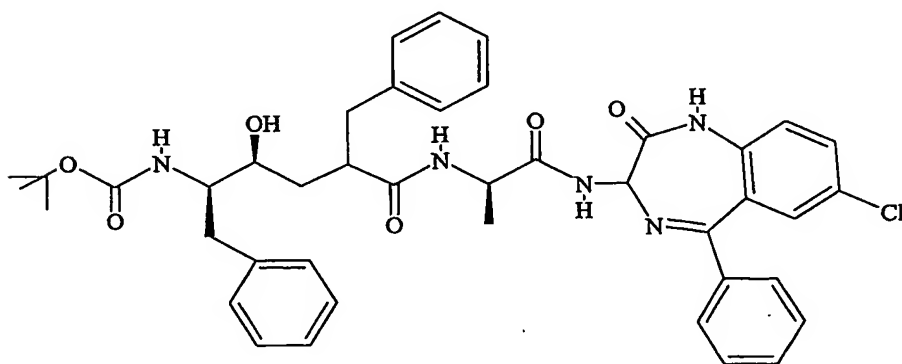
WPE-III-31C



DAPT



L852,646



NVP-AHW700-NX

Whatever the structure or the nature of the compounds identified by the methods of the present invention one can use the identified compounds to further develop compounds for therapeutic uses. Using lead compounds to help develop improved compounds is known as
5 “rational drug design” and includes not only comparisons with known inhibitors and activators, but predictions relating to the structure of target molecules.

The goal of rational drug design is to produce structural analogs of biologically active polypeptides or other compounds. By creating such analogs, it is possible to fashion drugs, which are more active or stable than the natural molecules, which have different susceptibility to
10 alteration or which may affect the function of various other molecules. In one approach, one would generate a three-dimensional structure for a target molecule, or a fragment thereof. This could be accomplished by x-ray crystallography, computer modeling or by a combination of both approaches.

It also is possible to use antibodies to ascertain the structure of a target compound
15 activator or inhibitor. In principle, this approach yields a pharmacore upon which subsequent drug design can be based. It is possible to bypass protein crystallography altogether by generating anti-idiotypic antibodies to a functional, pharmacologically active antibody. As a mirror image of a mirror image, the binding site of anti-idiotypic would be expected to be an analog of the original antigen. The anti-idiotypic could then be used to identify and isolate
20 peptides from banks of chemically- or biologically-produced peptides. Selected peptides would then serve as the pharmacore. Anti-idiotypes may be generated using the methods described herein for producing antibodies, using an antibody as the antigen.

On the other hand, one may simply acquire, from various commercial sources, small molecule libraries that are believed to meet the basic criteria for useful drugs in an effort to
25 “brute force” the identification of useful compounds. Screening of such libraries, including combinatorially generated libraries (*e.g.*, peptide libraries), is a rapid and efficient way to screen large number of related (and unrelated) compounds for activity. Combinatorial approaches also lend themselves to rapid evolution of potential drugs by the creation of second, third and fourth generation compounds modeled of active, but otherwise undesirable compounds.

Test compounds may include fragments or parts of naturally-occurring compounds, or
30 may be found as active combinations of known compounds, which are otherwise inactive. It is proposed that compounds isolated from natural sources, such as animals, bacteria, fungi, plant sources, including leaves and bark, and marine samples may be assayed as candidates for the presence of potentially useful pharmaceutical agents. It will be understood that the

pharmaceutical agents to be screened could also be derived or synthesized from chemical compositions or man-made compounds. Thus, it is understood that the candidate substance identified by the present invention may be peptide, polypeptide, polynucleotide, small molecule inhibitors or any other compounds that may be designed through rational drug design starting from known inhibitors or stimulators.

Other suitable modulators include antisense molecules, ribozymes, aptamers, and antibodies (including single chain antibodies), each of which would be specific for the target molecule. For example, an antisense molecule that bound to a translational or transcriptional start site, or splice junctions, would be ideal candidate inhibitors.

In addition to the modulating compounds initially identified, the inventors also contemplate that other sterically similar compounds may be formulated to mimic the key portions of the structure of the modulators. Such compounds, which may include peptidomimetics of peptide modulators, may be used in the same manner as the initial modulators.

b. *In vitro* Assays

Those of skill in the art will be familiar with a number of assays useful in evaluating a compound for the ability to inhibit γ -secretase activity. A quick, inexpensive and easy assay to run is an *in vitro* assay. Such assays generally use isolated molecules, can be run quickly and in large numbers, thereby increasing the amount of information obtainable in a short period of time. A variety of vessels may be used to run the assays, including test tubes, plates, dishes and other surfaces such as dipsticks or beads.

One example of a cell free assay is a binding assay. The ability of a modulator to bind to a target molecule in a specific fashion is strong evidence of a related biological effect. For example, binding of a molecule to an enzyme may block the catalysis of a substrate. Thus, a test compound could be screened for its ability to bind to PS1, APH-1, nicastrin, PEN-2, or APP. The interaction may be due to steric, allosteric or charge-charge interactions. The protein or protein complex may be either free in solution, fixed to a support, expressed in or on the surface of a cell. Either the protein or the compound may be labeled, thereby permitting determining of binding. Usually, the protein will be the labeled species, decreasing the chance that the labeling will interfere with or enhance binding. Competitive binding formats can be performed in which one of the agents is labeled, and one may measure the amount of free label versus bound label to determine the effect on binding.

In certain embodiments, a solubilized membrane preparation or γ -secretase isolated away from the solubilized membrane preparation from *Pichia pastoris* engineered to express one or more of PS1, APH-1, nicastrin, PEN-2, and APP is contacted with one or more test compounds, and the ability of the test compound(s) to alter γ -secretase activity is determined, as compared to a similar transgenic *P. pastoris* not treated with the test compound(s), to identify an inhibitor. In addition to γ -secretase activity, one may also determine other characteristics of the proteins that may be altered by the test compound. For example, the conversion of PS1 holoprotein to NTF/CTF could be determined. Alternatively, γ -secretase complex formation could be determined by, for example, co-immunoprecipitation assays.

A technique for high throughput screening of compounds is described in WO 84/03564. Large numbers of small peptide test compounds are synthesized on a solid substrate, such as plastic pins or some other surface. Bound polypeptide is detected by various methods.

d. *In vivo* Assays

In vivo assays of the invention involve the use of transgenic *Pichia pastoris* that have been engineered to express one or more of PS1, APH-1, nicastrin, PEN-2, and APP. Generally, the transgenic *P. pastoris* used for *in vivo* assays will co-express all five of the proteins listed above. In addition, it may be desirable to express a mutant protein. For example, PS1 M146L, E280A, G384A, or D385A may be expressed in place of, or along with, wild-type PS1.

In such assays, the transgenic *P. pastoris* is contacted with one or more test compounds, and the ability of the test compound(s) to alter γ -secretase activity is determined, as compared to a similar transgenic *P. pastoris* not treated with the test compound(s), to identify an inhibitor. In addition to γ -secretase activity, one may also determine other characteristics of the proteins that may be altered by the test compound. For example, the conversion of PS1 holoprotein to NTF/CTF could be determined. Alternatively, γ -secretase complex formation could be determined by, for example, co-immunoprecipitation assays.

The present invention provides methods of screening for a test compound that modulates γ -secretase activity. In these embodiments, the present invention is directed to a method for determining the ability of a test compound to inhibit γ secretase activity, generally including the steps of: contacting a test compound to the transgenic *P. pastoris*; and determining the ability of the test compound to inhibit γ -secretase activity.

The invention also contemplates *in vivo* studies involving various animal models to further characterize compounds identified as modulators of γ -secretase activity. Animal models

include transgenic animals that have been engineered to have specific defects, or carry markers that can be used to measure the ability of a compound to reach and effect different cells within the organism. Due to their size, ease of handling, and information on their physiology and genetic make-up, mice are a preferred embodiment, especially for transgenics. However, other
5 animals are suitable as well, including rats, rabbits, hamsters, guinea pigs, gerbils, woodchucks, cats, dogs, sheep, goats, pigs, cows, horses and monkeys (including chimps, gibbons and baboons). Assays for modulators may be conducted using an animal model derived from any of these species.

In such assays, one or more test compounds are administered to an animal, and the ability
10 of the test compound(s) to alter the level of γ -secretase activity are measured, as compared to a similar animal not treated with the test compound(s), identifies a modulator. In addition to γ -secretase activity, one may also measure other characteristics. These characteristics may be a change with regard to the function of a particular protein, *e.g.*, enzyme, receptor, hormone, ion channel, or instead a broader indication such as behavior, neurological response, physiological
15 response, pathological response, *etc.*

The present invention provides methods of screening for a test compound that modulates the activity of γ -secretase. In these embodiments, the present invention is directed to a method for determining the ability of a test compound to inhibit γ -secretase activity, generally including the steps of: administering a test compound to the animal; and determining the ability of the test
20 compound to reduce one or more characteristics of a disease or pathology caused by γ -secretase activity or symptoms associate with the same. In specific embodiments, the disease or pathology is Alzheimer's disease.

Treatment of these animals with test compounds will involve the administration of the compound, in an appropriate form, to the animal. Administration will be by any route that could
25 be utilized for clinical or non-clinical purposes, including but not limited to oral, nasal, buccal, or even topical. Alternatively, administration may be by intracerebral injection, intratracheal instillation, bronchial instillation, intradermal injection, subcutaneous injection, intramuscular injection, intraperitoneal injection or intravenous injection. Specifically contemplated routes are systemic intravenous injection, regional administration via blood or lymph supply, or directly to
30 an affected site.

Determining the effectiveness of a compound *in vivo* may involve a variety of different criteria. Also, measuring toxicity and dose response can be performed in animals in a more meaningful fashion than in *in vitro* or *in cyto* assays.

E. Clinical Trials

This section is concerned with the development of human treatment protocols for providing therapy to Alzheimer's disease (AD) and other conditions involving progressive dementia in a human patient using the γ -secretase inhibitors identified by the methods of the invention as described herein. Candidates for the phase 1 clinical trial will be patients on which all conventional therapies have failed. γ -secretase inhibitors described herein will be administered. Tests that will be used to monitor the progress of the patients and the effectiveness of the treatments include: physical exam, X-ray, blood work and other clinical laboratory methodologies. The doses given in the phase 1 study will be escalated as is done in standard phase 1 clinical phase trials, *i.e.* doses will be escalated until maximal tolerable ranges are reached. Clinical responses may be defined by acceptable measure. For example, a complete response may be defined by complete disappearance of the neurological disease or condition, whereas a partial response may be defined by about a 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or about a 95% reduction of the disease or condition. The typical course of treatment will vary depending upon the individual patient and disease being treated in ways known to those of skill in the art.

F. Immunodetection Methods

The present invention also concerns immunodetection methods for binding, purifying, removing, quantifying or otherwise generally detecting biological components. The steps of various useful immunodetection methods have been described in the scientific literature, such as, *e.g.*, Nakamura *et al.* (1987), incorporated herein by reference. Immunoassays, in their most simple and direct sense, are binding assays. Certain preferred immunoassays are the various types of enzyme linked immunosorbent assays (ELISAs), radioimmunoassays (RIA) and immunobead capture assay. Immunohistochemical detection using tissue sections also is particularly useful. However, it will be readily appreciated that detection is not limited to such techniques, and Western blotting, dot blotting, FACS analyses, and the like also may be used in connection with the present invention.

In general, immunobinding methods include obtaining a yeast cell transformed with an expression construct expressing a protein or peptide and contacting the sample with an antibody to the protein or peptide in accordance with the present invention, as the case may be, under conditions effective to allow the formation of immunocomplexes.

The immunobinding methods of this invention include methods for detecting or quantifying the amount of A β in a sample. Here, one would obtain a soluble membrane preparation from *P. pastoris* transformed with PS1, APH-1, PEN-2, and nicastrin, and contact the preparation with a substrate (e.g., APP C100Flag) with or without a test compound. An antibody to A β is then used to detect or quantify the amount of immune complexes formed under the specific conditions. Alternatively, one could obtain a *P. pastoris* cell transformed with PS1, APH-1, PEN-2, nicastrin, and APP or an APP derivative such as APP C99. The *P. pastoris* cell is contacted with a test compound and an antibody to A β is used to detect or quantify the amount of A β secreted from the cell.

Contacting the chosen biological sample with the protein, peptide or antibody under conditions effective and for a period of time sufficient to allow the formation of immune complexes (primary immune complexes) is generally a matter of simply adding the composition to the sample and incubating the mixture for a period of time long enough for the antibodies to form immune complexes with, i.e., to bind to, any antigens present. After this time, the sample-antibody composition, such as an ELISA plate, dot blot or Western blot, will generally be washed to remove any non-specifically bound antibody species, allowing only those antibodies specifically bound within the primary immune complexes to be detected.

In general, the detection of immunocomplex formation is well known in the art and may be achieved through the application of numerous approaches. These methods are generally based upon the detection of a label or marker, such as any radioactive, fluorescent, biological or enzymatic tags or labels of standard use in the art. U.S. Patents concerning the use of such labels include 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149 and 4,366,241, each incorporated herein by reference. Of course, one may find additional advantages through the use of a secondary binding ligand such as a second antibody or a biotin/avidin ligand binding arrangement, as is known in the art.

The encoded protein, peptide or corresponding antibody employed in the detection may itself be linked to a detectable label, wherein one would then simply detect this label, thereby allowing the amount of the primary immune complexes in the composition to be determined.

Alternatively, the first added component that becomes bound within the primary immune complexes may be detected by means of a second binding ligand that has binding affinity for the encoded protein, peptide or corresponding antibody. In these cases, the second binding ligand may be linked to a detectable label. The second binding ligand is itself often an antibody, which may thus be termed a "secondary" antibody. The primary immune complexes are contacted with the labeled, secondary binding ligand, or antibody, under conditions effective and for a period of

time sufficient to allow the formation of secondary immune complexes. The secondary immune complexes are then generally washed to remove any non-specifically bound labeled secondary antibodies or ligands, and the remaining label in the secondary immune complexes is then detected.

5 Further methods include the detection of primary immune complexes by a two step approach. A second binding ligand, such as an antibody, that has binding affinity for the encoded protein, peptide or corresponding antibody is used to form secondary immune complexes, as described above. After washing, the secondary immune complexes are contacted with a third binding ligand or antibody that has binding affinity for the second antibody, again
10 under conditions effective and for a period of time sufficient to allow the formation of immune complexes (tertiary immune complexes). The third ligand or antibody is linked to a detectable label, allowing detection of the tertiary immune complexes thus formed. This system may provide for signal amplification if this is desired.

15 E. Pharmaceutical Preparations

Pharmaceutical compositions of the present invention comprise an effective amount of one or more γ -secretase inhibitors identified as described by the present invention dissolved or dispersed in a pharmaceutically acceptable carrier. The phrases "pharmaceutical" or "pharmacologically acceptable" refer to molecular entities and compositions that do not produce
20 an adverse, allergic or other untoward reaction when administered to an animal, such as, for example, a human, as appropriate. The preparation of an pharmaceutical composition that contains at least one γ -secretase inhibitor or additional active ingredient will be known to those of skill in the art in light of the present disclosure, as exemplified by Remington's Pharmaceutical Sciences, 1990, incorporated herein by reference. Moreover, for animal (*e.g.*,
25 human) administration, it will be understood that preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biological Standards.

As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, antioxidants, salts, coatings, surfactants, preservatives (*e.g.*, methyl or propyl
30 p-hydroxybenzoate, sorbic acid, antibacterial agents, antifungal agents), isotonic agents, solution retarding agents (*e.g.* paraffin), absorbents (*e.g.* kaolin clay, bentonite clay), drug stabilizers (*e.g.* sodium lauryl sulphate), gels, binders (*e.g.* syrup, acacia, gelatin, sorbitol, tragacanth, polyvinylpyrrolidinone, carboxy-methyl-cellulose, alginates), excipients (*e.g.* lactose, milk sugar, polyethylene glycol), disintegration agents (*e.g.* ager-ager, starch, lactose, calcium

phosphate, calcium carbonate, alginic acid, sorbitol, glycine), wetting agents (*e.g.* cetyl alcohol, glycerol monostearate), lubricants, absorption accelerators (*e.g.* quaternary ammonium salts), edible oils (*e.g.* almond oil, coconut oil, oily esters or propylene glycol), sweetening agents, flavoring agents, coloring agents, fillers, (*e.g.* starch, lactose, sucrose, glucose, mannitol, silicic acid),
5 tableting lubricants (*e.g.* magnesium stearate, starch, glucose, lactose, rice flower, chalk), carriers for inhalation (*e.g.* hydrocarbon propellants), buffering agents, or such like materials and combinations thereof, as would be known to one of ordinary skill in the art (see, for example, Remington's Pharmaceutical Sciences, 1990, incorporated herein by reference). Except insofar
10 as any conventional carrier is incompatible with the active ingredient, its use in the therapeutic or pharmaceutical compositions is contemplated.

In any case, the composition may comprise various antioxidants to retard oxidation of one or more component. Examples of antioxidants includes ascorbic acid, cysteine hydrochloride, sodium sulfite, sodium bisulfite, sodium metabisulfite, ascorbyl palmitate, butylated hydroxytoluene, butylated hydroxyanisole, lecithin, propyl gallate, and -tocopherol.
15 Additionally, the prevention of the action of microorganisms can be brought about by preservatives such as various antibacterial and antifungal agents, including but not limited to parabens (*e.g.*, methylparabens, propylparabens), chlorobutanol, phenol, sorbic acid, thimerosal or combinations thereof).

The γ -secretase inhibitor may be formulated into a composition in a free base, neutral or salt form. Pharmaceutically acceptable salts, include the acid addition salts, *e.g.*, those formed
20 with the free amino groups of a proteinaceous composition, or which are formed with inorganic acids such as for example, hydrochloric, hydrobromic, or phosphoric acids; or such organic acids as acetic, oxalic, tartaric, benzoic, lactic, phosphoric, citric, maleic, fumaric, succinic, tartaric, napsylic, clavulanic, stearic, or mandelic acid. Salts formed with the free carboxyl groups can
25 also be derived from inorganic bases such as for example, sodium, potassium, ammonium, calcium magnesium or ferric hydroxides; or such organic bases as isopropylamine, trimethylamine, histidine or procaine.

In embodiments where the composition is in a liquid form, a carrier can be a solvent or dispersion medium comprising but not limited to, water, ethanol, polyol (*e.g.*, glycerol, propylene glycol, liquid polyethylene glycol, *etc.*), lipids (*e.g.*, triglycerides, vegetable oils,
30 liposomes) and combinations thereof. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin; by the maintenance of the required particle size by dispersion in carriers such as, for example liquid polyol or lipids; by the use of surfactants such as, for example hydroxypropylcellulose; or combinations thereof such methods. In many cases, it will

be preferable to include isotonic agents, such as, for example, sugars, sodium chloride or combinations thereof.

The γ -secretase inhibitor may also comprise different types of carriers depending on whether it is to be administered in solid or liquid form, and whether it need to be sterile for such routes of administration as injection. The present invention can be administered orally, intradermally, subcutaneously, topically, by injection, infusion, continuous infusion, localized perfusion, bathing target cells directly, *via* a catheter, via a lavage, or by other methods or any combination of the forgoing as would be known to one of ordinary skill in the art (see, for example, Remington's Pharmaceutical Sciences, 1990, incorporated herein by reference).

The γ -secretase inhibitor when administered orally may be in the form of tablets, capsules, sachets, vials, powders, granules, lozenges, reconstitutable powders, liquid preparations. The γ -secretase inhibitor may be administered via transdermal delivery using a skin-patch formulation. The γ -secretase inhibitor may be dispersed in a pressure sensitive adhesive which adheres to the skin such that it can diffuse through the skin for delivery to the patient.

Transdermal adhesives such as natural rubber or silicone are known in the art.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filter sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle that contains the basic dispersion medium and/or the other ingredients. In the case of sterile powders for the preparation of sterile injectable solutions, suspensions or emulsion, the preferred methods of preparation are vacuum-drying or freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered liquid medium thereof. The liquid medium should be suitably buffered if necessary and the liquid diluent first rendered isotonic prior to injection with sufficient saline or glucose. The preparation of highly concentrated compositions for direct injection is also contemplated, where the use of DMSO as solvent is envisioned to result in extremely rapid penetration, delivering high concentrations of the active agents to a small area.

The actual dosage amount of a composition of the present invention administered to a patient can be determined by physical and physiological factors such as body weight, gender, severity of condition, the type of disease being treated, previous or concurrent therapeutic interventions, idiopathy of the patient, time of the administration, rate of excretion of the particular compound, and on the route of administration. The practitioner responsible for administration will, in any event, determine the concentration of active ingredient(s) in a

composition and appropriate dose(s) for the individual subject. The dosage will also depend upon the bioavailability and activity of the particular γ -secretase inhibitor.

The composition must be stable under the conditions of manufacture and storage, and preserved against the contaminating action of microorganisms, such as bacteria and fungi. It will be appreciated that endotoxin contamination should be kept minimally at a safe level, for example, less than 0.5 ng/mg protein.

In particular embodiments, prolonged absorption of an injectable composition can be brought about by the use in the compositions of agents delaying absorption, such as, for example, aluminum monostearate, gelatin or combinations thereof.

X. Examples

The following example is included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

Example 1

Construction of Expression Plasmids Under the Control of AOX1 Promoter

The wild-type PS 1 expression plasmid was generated as follows. Wild-type PS1 (PS1WT) was released from pAG3-PS1WT by restriction enzymes KpnI and BamH I, and inserted into pIB4 vector at the corresponding sites to generate pIB4-PS1WT.

The PS1 D385A mutant expression plasmid, pIB4-PS1D385A, was similarly constructed with pAG3-PS1D385A and pIB4 vectors.

To generate PS1 with a Tandem Affinity Purification (TAP) tag, a 500bp TAP fragment was released from p1761 vector with enzymes Hind III and Nco I (the Nco I site was blunted with Klenow), and a second fragment encoding wild-type PS1 was isolated from pAG3-TAP PS1 WT digested with Hind III and BamH I. These fragments were then ligated with pIB4 vector, which was already cut with Sma I and BamH I, to generate pIB4-TAP PS1 WT.

The PS1 M146L mutant expression plasmid, pIB4-TAP PS1 M146L, was constructed as follows. A fragment containing the PS1 M146L mutation was released from pAG3-PS1 M146L by SnaB I and BamH I. This fragment was then ligated with pIB4-TAP PS1 WT, which was

digested with enzyme SnaB I and BamH I. The same strategy was used to construct the following expression plasmids: pIB4-TAP PS1 E280A, pIB4-TAP PS1 G384A, pIB4-TAP PS1 D385A, pIB4-PS1 M146L, pIB4-PS1 E280A, pIB4-PS1 G384A, pIB4-PS1 D385A.

The APH-1 expression plasmid was generated as follows. Two primers were used to
5 PCRTM amplify a 900bp fragment encoding APH1 α L fused to c-myc and hexahistidine from the vector pcDNA4-APH1 α L (from Dr. G. Yu, University of Texas Southwestern Medical Center at Dallas). The forward primer is APH1 α EI Fr: GGC GAA TTC ACC ATG GGG GCT GCG GTG T (SEQ ID NO:3); and the reverse primer is HISstop EI Re: GGC GAA TTC TCA ATG GTG ATG GTG ATG ATG (SEQ ID NO:4). The amplified DNA fragment was digested with
10 EcoR I and ligated into a vector pBLURA-IX linearized with EcoR I. The resulting plasmid is pBLURAIIX-APH1. The sequence of the region amplified with PCRTM was confirmed by sequence reaction analysis.

The nicastrin expression plasmid was generated as follows. Nicastrin cDNA was PCRTM amplified from pAG3-NCT plasmid with a forward primer NCT Mfe Fr: GCC CAATTG ACC
15 ATG GCT ACG GCA GGG GGT (SEQ ID NO:5); and a reverse primer NCT Mfe Re: GCC CAATTG TCA GTA TGA CAC AGC TCC TGG (SEQ ID NO:6). The PCRTM product was digested with Mfe I and inserted into the vector pBLADE-IX linearized with EcoR I. This resulted in a plasmid pBLADEIX-NCT. NCT sequence was confirmed by sequencing reaction.

The PEN-2 expression plasmid was generated as follows. PEN2 cDNA was PCRTM amplified from the plasmid pAG3-PEN2 with a forward primer PEN2 EI Fr: GGC GAA TTC
20 ACC ATG AAC CTG GAG CGA GTG (SEQ ID NO:7), and a reverse primer PEN2 EI Re: GGC GAA TTC TCA GGG GGT GCC CAG GGG TAT (SEQ ID NO:8). The PCRTM product was digested with EcoR I and inserted into the vector pBLADE-IX linearized with EcoR I. This resulted in a plasmid pBLARGIX-PEN2. PEN-2 sequence was confirmed by sequencing
25 reaction.

The APP C99 expression plasmid was generated as follows. The APP C99 cDNA was amplified from pCB6-APP^{swe} by PCRTM with forward primer TCTCTCGAGAAAAGAGATGCAGAATTCCGACATGAC (SEQ ID NO:9) and reverse primer GGCTCTAGACCGTTCTGCATCTGCTCAAAGAAC (SEQ ID NO:10). The PCRTM
30 product was digested with XhoI and XbaI and inserted into the Pichia expression vector pGAP α A linearized with XhoI and XbaI. This resulted in a plasmid pGAPZ α A-CTF. The APP C99 sequence was confirmed by sequencing reaction. The protein encoded by the pGAPZ α A-CTF plasmid contains the yeast α -factor signal sequence fused to APP C99 followed by a myc-6XHis epitope tag.

PEP4 promoter DNA was amplified from *Pichia* genomic DNA by PCR™ with forward primer Ppep4Fr2 GCCAGATCTCATCTTGTGACTGGTTGATC (SEQ ID NO:11) and reverse primer Ppep4Re CGCGGATCCGTAAGTACCGTCAAATATCATC (SEQ ID NO:12). The PCR™ product was digested with Bgl II and BamHI and inserted into the APP C99 expression plasmid pGAPZαA-CTF linearized with Bgl II, resulting in a plasmid pGAPZαA-CTF-Ppep4.

PEP4 terminator DNA was amplified from *Pichia* genomic DNA by PCR™ with forward primer Tpep4Fr CGCGGATCCAGGTAGTGAAGATGTAGAG (SEQ ID NO:13) and reverse primer Tpep4Re (SEQ ID NO:14) GGCAGATCTGAGCCATACTTCCAACATC. The PCR™ product was digested with Bgl II and BamHI and inserted into the APP C99 expression plasmid pGAPZαA-CTF-Ppep4 linearized with Bgl II, resulting in a plasmid pGAPZαA-CTF-PEP4KO. This plasmid was linearized with Bgl II and integrated into PEP4 genomic locus.

Example 2

Generation of Transgenic *Pichia pastoris*

The *P. pastoris* strain JC308 (*adel1 arg4 his4 ura3*) was used as a parent strain. Yeast transformation was performed according to the procedure of Sears *et al.* (1998). For double integration at two loci in a single transformation reaction, 4 μg of DNA of each plasmid was used. pIB4-PS1 expression plasmid series was linearized with EcoN I to integrate plasmids into the *HIS4* locus. pBLURAIX-APH1αL was digested with Sal I to integrate the plasmid into the *URA3* locus, pBLADEIX-NCT with Spe I digestion to integrate into the *ADE1* locus, pBLARGIX-PEN2 with Avr II digestion to integrate into the *ARG4* locus, and pGAPZαA-CTF-PEP4KO with Bgl II digestion to integrate at the *PEP4* locus. The selection markers were *His4* for PS1, *Ura3* for APH-1, *Adel1* for NCT, *Arg4* for PEN-2, and *Sh ble Zeocin®* for APP C99.

Integrations of plasmids encoding APH-1, PS1, PEN-2, or NCT in the *P. pastoris* genome were confirmed by PCR™ analysis. An ~1100 bp fragment of APH-1 was amplified with primers 5' AOX1 and 6xHis Re. A 400 bp fragment of PS1 was amplified with primers BamH I L365Fr and 3' AOX1. A 400bp fragment of PEN-2 was amplified with primers PEN2Fr and 3'AOX1. A 1600 bp fragment of NCT was amplified with primers 5'AOX1 and GST As.

Example 3

Induction of γ-Secretase Expression in *P. pastoris*

All *P. pastoris* strains were grown in YPD or SYD medium overnight, and then diluted to 1:1000 in BMGY medium for cells to reach the log phase. These cells were collected by

centrifugation at 5000x g for 5 minutes, resuspended in sterile water, and then collected again. Cells were diluted to OD600 = 1 in the induction medium BMMY with 1% Casamino acid. Cells were harvested after 0, 3, 6, and 12 hours of induction. Cells were then broken with 500 μ m acid-washed glass beads in Buffer B (50mM MES, pH6.0, 150mM KCl, 5mM CaCl₂, 5mM MgCl₂) with protease inhibitors (Roche), 1mM PMSF, and 1 μ M Pepstatin. Microsome membranes were harvested from post-nuclear supernatant by centrifugation under 100,000x g for 1 hour. Expression of PS1, A Φ H-1, nicastrin, and PEN-2 was verified by western blot analysis (FIG. 1). The antibody PS1 NT was used to detect PS1 holoprotein and PS1 NTF, the antibody PS1 Loop was used to detect PS1 holoprotein and PS1 CTF, the antibody PNT-2 was used to detect PEN-2, the antibody NCT #54 was used to detect nicastrin, and a commercial monoclonal c-myc antibody, 9E10, was used to detect A Φ H-1 (a myc tag was fused to the C-terminus of A Φ H-1).

Example 4

Conversion of PS1 Holoprotein to NTF/CTF

All *P. pastoris* strains were grown in YPD or SYD medium overnight, and then diluted to 1:1000 in BMGY medium for cells to reach the log phase. These cells were collected by centrifugation at 5000x g for 5 minutes, resuspended in sterile water, and then collected again. Cells were diluted to OD600 = 1 in the induction medium BMMY with 1% Casamino acid. Cells were harvested after 5 hours of induction. Cells were then broken with 500 μ m acid-washed glass beads in Buffer B (50mM MES, pH6.0, 150mM KCl, 5mM CaCl₂, 5mM MgCl₂) with protease inhibitors (Roche), 1mM PMSF, and 1 μ M Pepstatin. Microsome membranes were harvested from post-nuclear supernatant by centrifugation under 100,000x g for 1 hour. Conversion of the PS1 holoprotein to PS1 NTF and PS1 CTF was verified by western blot analysis (FIG. 2). The antibody PS1 NT was used to detect PS1 holoprotein and PS1 NTF.

Example 5

In Vitro γ -Secretase Activity

The γ -secretase assay was modified from the procedure by Li *et al.* (2000), with 0.1% phosphatidylcholine (PC) and 0.05% phosphatidylethanolamine (PE) in Buffer A to solubilize γ -secretase from membrane preparations, and with 0.1% PC and 0.025% PE in final reaction. Soluble membrane preparations were made from *P. pastoris* strains expressing various combinations of PS1, A Φ H-1, PEN-2, and nicastrin, as indicated in FIG. 3. Soluble membrane preparations from 293 cells were included as a positive control. The substrate APP C100Flag

was added into the soluble membrane preparation. The γ -secretase cleavage products CTF γ and A β were analyzed by western blot (FIG. 3). The antibody 26D6 was used to detect APP C100Flag and A β , and the CT15 antibody for APP C100Flag and CTF γ . CTF γ and A β were detected only in the 293 cells and *P. pastoris* strains co-expressing PS1, APh-1, PEN-2, and nicastrin.

Example 6

Inhibition of γ -Secretase Activity

The γ -secretase assay was modified from the procedure by Li *et al.* (2000), with 0.1% phosphatidylcholine (PC) and 0.05% phosphatidylethanolamine (PE) in Buffer A to solubilize γ -secretase from membrane preparations, and with 0.1% PC and 0.025% PE in final reaction. Soluble membrane preparations were made from *P. pastoris* strains expressing various combinations of PS1, APh-1, PEN-2, and nicastrin, as indicated in FIG. 3. Soluble membrane preparations from 293 cells were included as a positive control. The substrate APP C100Flag and the γ -secretase inhibitor L685,458 were added into the soluble membrane preparation. The γ -secretase cleavage products CTF γ and A β were analyzed by western blot (FIG. 3). The antibody 26D6 was used to detect APP C100Flag and A β , and the CT15 antibody for APP C100Flag and CTF γ . The presence of CTF γ and A β was greatly reduced in the soluble membrane preparations treated with L685,458 as compared to preparations without L685,458.

Example 7

γ -Secretase Activity *In Vivo*

To evaluate γ -secretase activity *in vivo*, the inventors created a *P. pastoris* JC308 (*adel arg4 his4 ura3*) strain in which a cDNA (SEQ ID NO: 15) encoding an epitope-tagged APP C99 polypeptide (SEQ ID NO: 16) is stably integrated into the *PEP4* locus. The yeast were also modified as described in Example 2 to express PS1, APh-1, nicastrin, and PEN-2. Cells were cultured in BMGY to mid-log phase. Cells were then either inoculated into BMGY or BMMY with 1% casamino acids at OD600 = 1. Culture in BMMY induces the expression of PS1, APh-1, nicastrin, and PEN-2. Cells were harvested 5 hours after induction. The conditioned media was also collected for analysis.

Integration of the APP C99 construct at the *PEP4* locus inactivates the principal protease system in *P. pastoris*, and permits the assessment of the levels and identities of secreted A β and intracellular AICD (amyloid intracellular domain) derivatives *in vivo*. The encoded protein

contains the yeast α -factor signal sequence fused to APP C99 followed by a myc-6XHis epitope tag. The α -factor signal sequence efficiently targets APP C99 into the intracellular membrane, but it is cleaved from the fusion protein after translocation into the ER, resulting in a C99 fusion protein without this signal sequence. The myc-6XHis facilitates the detection and purification
5 for this fusion protein. Processing of the mata-APP C99 fusion protein by the yeast KEX2 protease immediately carboxyl to the dibasic lys-arg sequence (amino acids 84 and 85 of SEQ ID NO: 16) is expected to generate an amino-terminus A β fragment and a carboxyl-terminus C99 fragment.

As shown in FIG. 4A, the expression of the mata-APP C99 fusion protein and the
10 KEX2-processed C99 polypeptides was detected in cell lysates by western blot analysis using a myc-specific 9E10 antibody. Lane 1 of FIG. 4A was loaded with lysate from cells in which the γ -secretase complex was not induced. Upon induction of the expression of γ -secretase components (lane 2), the inventors observed the production of a carboxyl-terminal, myc-specific fragment of ~6 kDa, that likely represents CTF γ . Parallel analysis of the conditioned medium
15 from uninduced cultures (FIG. 4A, lane 3) and induced cultures (FIG. 4A, lane 4) reveals specific production of 4G8-immunoprecipitable A β peptides.

A parallel cell lysate as that shown in FIG. 4A, lane 2 was subject to centrifugation at 100,000 x g for 1 hour to separate membrane and soluble proteins. Proteins were fractionated on Tris-Tricine gels, and Western blotting was performed with myc-specific 9E10 and A β -specific
20 26D6 antibodies. In the total post-nuclear supernatant (T) (FIG. 4A, lane 5), the inventors observed both CTF γ and A β . However, only A β was observed in the 100,00 x g membrane pellet (P) (FIG. 4A, lane 6), as expected if the peptide was generated in intracellular vesicles. Similarly, CTF γ was only detected in the 100,000 x g supernatant (S) (FIG. 4A, lane 7), as
25 would be expected if the fragment was released from membranes after cleavage at the "e-cleavage" site.

To confirm the identity of A β peptides generated in this experiment, the inventors performed immunoprecipitation-mass spectrometry analysis (IP-MS). Protein samples as shown in FIG. 4A were immunoprecipitated with antisera 4G8, and subjected to MALDI-TOF mass spectrometry analysis (FIG. 4B). As expected, the inventors did not observe production of A β
30 peptides under conditions where the γ -secretase components are in basal, uninduced conditions. However, the inventors observed production of A β 3-40 and A β 3-42 and shorter A β derivatives in cell lysates upon induction of the γ -secretase components. The inventors also observed low levels of A β species in the pellet fraction, but undetectable levels in the supernatant fraction

from the samples shown respectively in FIG. 4A, lane 7 and lane 6. Finally, A β peptide variants were detected in the conditioned medium from induced cells, but not in the conditioned medium from uninduced cells. The finding that all A β peptide species begin at +3 was not anticipated. However, after KEX2 cleavage (after the dibasic residues), the yeast protease STE13 is known to
5 cleave dipeptides containing the sequence Glu Ala. Therefore, the first two amino acids (Asp Ala) in A β are very likely being liberated by STE13, resulting in A β species that start at the +3 position.

Collectively, these studies demonstrate that in *P. pastoris* strains that over express all four components of the γ -secretase complex, a constitutively expressed C99 polypeptide is
10 processed in a manner very similar to that observed in mammalian cells. This system now allows us to examine the effects of FAD-linked PS1 and APP mutants on processing and more importantly, offers a highly reliable and robust system to examine the structural and functional requirements of individual components of the complex on γ -secretase processing. Moreover, this provides a system in which γ -secretase inhibitors can be tested in an *in vivo* setting.

15 Example 8

Method of Screening for Inhibitors of γ -Secretase Activity

Soluble membrane preparations made from *P. pastoris* strains expressing PS1, APH-1, PEN-2, and nicastrin may be used to screen for inhibitors of γ -secretase activity. The γ -secretase assay may be modified from the procedure by Li *et al.* (2000), with 0.1% phosphatidylcholine
20 (PC) and 0.05% phosphatidylethanolamine (PE) in Buffer A to solubilize γ -secretase from membrane preparations, and with 0.1% PC and 0.025% PE in final reaction. A test compound can be added to the soluble membrane preparation along with a γ -secretase substrate, *e.g.*, APP C100Flag. The presence of a γ -secretase cleavage product could then be determined by western blot or electrochemiluminescence (ECL) assay.

25 For example, the ECL assay may be performed as follows. A β 40 can be selectively separated from the soluble membrane preparation by a specific antibody. A second antibody ruthenylated with TAG-N-hydroxysuccinamide ester recognizing only A β 40 can then be added to bind A β 40. The ruthenylated antibody will emit light under laser light to give an arbitrary unit for the amount of A β 40. By comparing the amount of light emitted in the assay with the amount
30 of light emitted from a series of known amounts of A β 40, the quantity of A β 40 generated in the assay can be determined. Similarly, a ruthenylated A β 42 antibody can be used to measure the

amount of $A\beta_{42}$. A decrease in the amount of $A\beta$ generated in the presence of the test compound would indicate that the compound is an inhibitor of γ -secretase activity.

* * * * *

5 All of the methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and
10 scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

REFERENCES

The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference.

- 5 U.S. Patent 3,817,837
U.S. Patent 3,850,752
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